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Pathways of Calcium Penetration through Isolated Cuticles of 'Golden Delicious' Apple Fruit

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Abstract. **Cuticles from 'Golden Delicious' apples (*Malus domestica* Borkh.) were isolated enzymatically. Changes in cuticles of developing fruit at various stages were studied using scanning electron (SEM) and light microscopy (LM). Structures allowing Ca penetration through the cuticle were studied by precipitating permeating Ca in agar as it passed through the cuticle. The importance of lenticels in the penetration of Ca at 2 stages of fruit development was studied under saturated conditions using a diffusion cell. The lenticels had a significant positive effect on Ca penetration. The development of a dense network of structures resembling cracks, as well as occasional surface discontinuities, were observed in cuticles isolated from fruit harvested in September. These structures seemed to provide pathways for cuticular Ca penetration. The importance of lenticels, cracks, or other surface irregularities, and the process of diffusion in the penetration of Ca into apple fruit is discussed.**

Calcium has been found to play an important role in maintaining fruit quality and prolonging storage life (1, 2, 5, 21). Spray applications of Ca during fruit development provide a safe method of supplementing endogenous Ca. Such treatments have been shown to reduce the incidence of physiological disorders, maintain fruit firmness, and reduce the rate of senescence (6, 18, 19, 20).

The major barrier to penetration of Ca sprays into the fruit is the cuticle (16, 26). A disadvantage of Ca spray applications when compared to postharvest Ca treatments is that several applications are needed to increase fruit Ca levels significantly (2, 29). Since little or no subsequent translocation of Ca from the leaves to the fruit occurs, Ca sprays must be applied directly to the fruit surface to be effective (11, 22). Late season spray applications are more effective than early season sprays in increasing the Ca levels of the fruit (4, 5, 11, 25). This difference may be partly due to the fact that the total surface area of a fruit is greatest in the fall, thus providing a greater area for penetration. Another explanation offered by Link (11) is that some Ca actually moves out of fruit sprayed early in the season. However, increased penetration through the cuticle in the latter stages of development also could account for greater effectiveness of fall sprays. An understanding of the mechanisms involved in Ca penetration would enhance efforts to make Ca applications more effective.

The purpose of this study was to investigate the pathways, and the relative importance of each pathway, of Ca penetration into apple fruit using isolated apple cuticles.

Materials and Methods

Golden Delicious apples were harvested each month from mid-June to mid-October of 1981 and 1982 from the horticulture orchards located in Pullman. Cuticles were isolated as described by Orgell (17). Cuticles of fruit harvested in June were difficult to isolate enzymatically due to the density of the tissue and to the difficulty in removing substantial amounts of cortical tissue before isolation. Therefore, after an incubation period of 72 hr, the cuticles were stripped from the fruit. Scanning electron micrographs revealed a layer of epidermal cells that remained attached to the cuticle. Cuticles from subsequent stages of fruit development were isolated enzymatically, and were found to be free of residual epidermal cells.

Scanning electron micrographs were prepared from isolated cuticles fixed in a 3% glutaraldehyde solution (buffered in 0.1 M phosphate buffer). The samples were dehydrated in a standard ethanol series, critical point dried, and sputter coated with gold-palladium. Samples were viewed in the SEM (International Scientific Instrument model 60).

Cuticle thickness was measured using an ocular micrometer and a calibrated stage micrometer with the aid of a light microscope. Thickness was measured as the distance between the inner periclinal wall of the epidermal cell wall and the outer cuticular surface. The mean thickness of 25 readings was taken for each stage of development.

Changes in cuticular permeability were measured at 3 stages of development using a procedure similar to that of McFarlane and Berry (13). In brief, 32 cuticles of each stage of development were mounted in a diffusion cell. A 0.5 M donor solution of $CaCl_2$ was flowed across the outer surface of the cuticles while a collection solution of NaCl having the same concentration was flowed across the inner surface of the cuticle. Permeating Ca was collected and analyzed to determine the permeability coefficients of cuticles at various stages of development. The effect of lenticels on permeability was measured by determining the difference between the permeability coefficients of cuticles with and without lenticels exposed. Values for the permeability of cuticles from apples harvested in July with no lenticels exposed were unattainable because of the dense distribution of lenticels. Diffusion studies comparing cuticles with and or without cracks was not considered feasible due to the delicate nature of the cracked cuticles which tended to detach along the cracks. Localization studies therefore were used to assess the relative importance of cracks and other surface irregularities.

Calcium penetration through the cuticles was localized by applying a Ca solution onto the outer surface of agar mounted cuticles and precipitating the permeating Ca on the agar surface.

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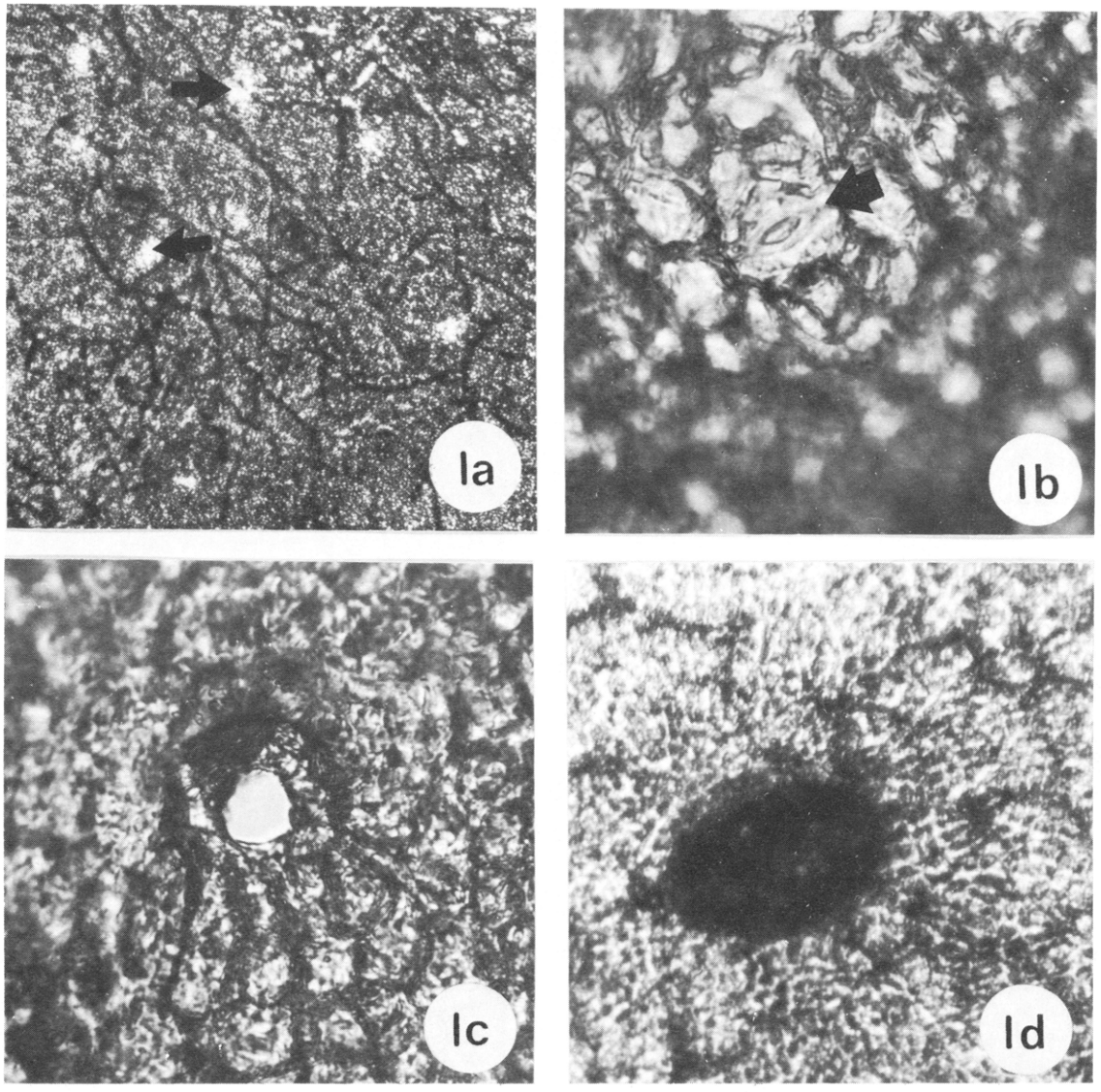


Fig. 1. **A**. Distribution of stoma (arrows) on the surface of a cuticle from fruits harvested in mid-June. x28 Magnification. **B**. Stomatal structure (arrow) on the surface of a cuticle from fruits harvested in mid-June. Magnification x63. **C**. Distorted stomatal structure occurring on a cuticle from fruit harvested in July. Magnification x56. **D**. Lenticel (dark region) on the surface of a cuticle from fruits harvested in September. Periderm formation of the lenticel acts as a protective covering similar to the cuticle and may function in gas exchange. Note the occurence of holes in the lenticel. Magnification x45.

This was accomplished by sealing the periphery of isolated cuticle disks with wax exposing 0.635 cm^2 of surface area of the center of the disk. A 1% molten agar solution was prepared and poured into a plexiglas tray to a depth of 0.6 cm. The cuticles were mounted on the molten agar when it had cooled to 30°C. Once the agar had set, disks of agar containing the cuticle were cut out using a 2.3 cm cork borer designed for this purpose. A saturated solution of oxalic acid was poured around the agar disks and allowed to diffuse throughout the agar. A 0.1 ml drop of 4% $CaCl_2$ solution was applied to each cuticle surface. Polarized light microscopy was used to localize Ca oxalate crystals formed on the agar surface by the permeating Ca ions.

Results

Light micrographs revealed evidence of lenticel development from stomata, similar to earlier claims (30). Cuticles from fruit harvested in June showed a dense distribution of stoma on the fruit surface (Fig. 1a, 1b). The greatest density of stoma occurred on the calyx end of the fruit. By July, many of the stomatal structures appeared distorted and open (Fig. 1c). The surface structures observed in cuticles from fruit collected in August scarcely resembled stomata but rather appeared as lenticels exhibiting periderm formation (Fig. 1d). This was confirmed by studies using cross sections of epidermal tissue.

The lenticels appeared to the unaided eye as small white areas on the surface of intact fruit harvested in August. In October, small white lenticels were still prominent on the calyx end of the fruit with a density of 28 ± 4 lenticels/cm^2. On the stem end of the fruit the lenticels seemed larger than those on the calyx end, were more sparsely distributed (8 ± 3 lenticels/cm^2), and exhibited brown corky tissue formation on the surface. The number of lenticels observed per fruit ranged from 1000 to 1200.

The cuticles from fruit harvested in September had irregularities, such as russetting, and the appearance of surface cracks.

Although the structures resembling cracks at times appeared irregular (Fig. 9), often a reticulate pattern was observed (Fig. 8b).

The thickness of the cuticle increased sharply between June and July (Fig. 2). Scanning electron micrographs of cuticles isolated from fruit harvested in June and July revealed large changes in both the topography of the cell surface and the formation of epicuticular wax structures (Figs. 3 and 4).

During fruit growth, cuticular pegs or extensions developed into the areas between the epidermal cell walls. The development of cuticlar pegs was not noticeable until July. The rate of increase in cuticular thickening leveled off after fruit was stored for 4 months.

Scanning electron micrographs of cuticles at various stages of fruit development were taken to observe changes in the cuticle surface and wax deposition. Since one step of the SEM procedure involved an alcohol dehydration series, the fine structure of the cuticle may have been altered. Nevertheless, the pictures were useful in observing the wax deposition and the gross morphology of the cuticles. Large changes in epicuticular wax deposition were observed from June to August (Fig. 3 and 5), even though these changes may have been decreased due to extraction. Changes in surface topography appeared to begin in June and became more prominent with each stage of development. Measurements of the cell size of the epidermis were found to correlate well with the size of the surface structures formed, suggesting that each structure most likely corresponds to one epidermal cell.

Studies of cuticular permeability with no lenticels exposed

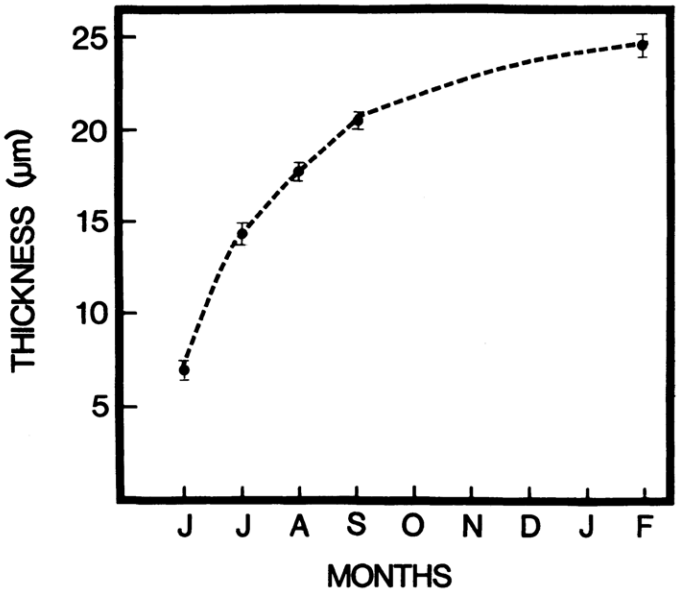


Fig. 2. Time course showing the increase in cuticular thickness during each stage of development. Bars indicate SE of mean.

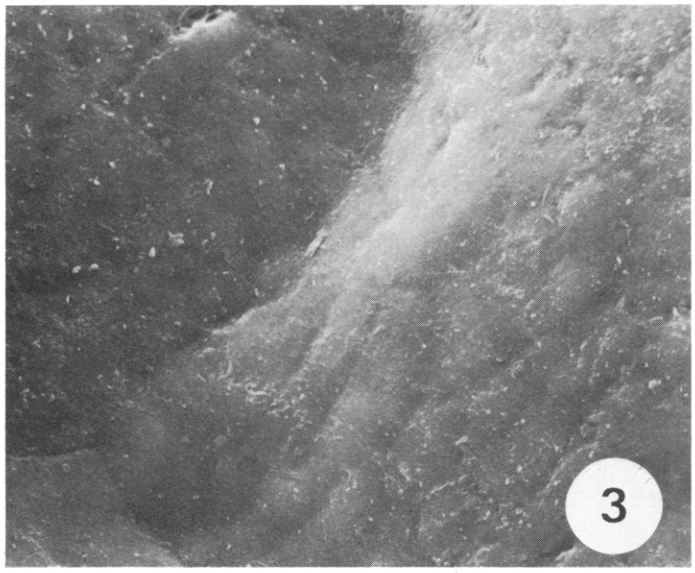


Fig. 3. Scanning electron micrograph of a cuticle from an apple fruit harvested in June. Note that the surface is relatively smooth with little or no visible fine wax structure. Magnification x650.

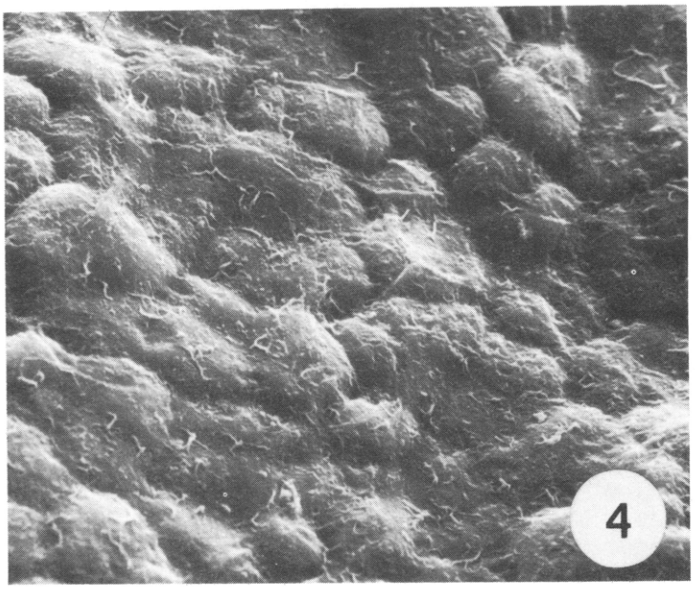


Fig. 4. Scanning electron micrograph of a cuticle from an apple fruit harvested in July. Note the development of the surface topography, fine wax structures. Magnification x650.

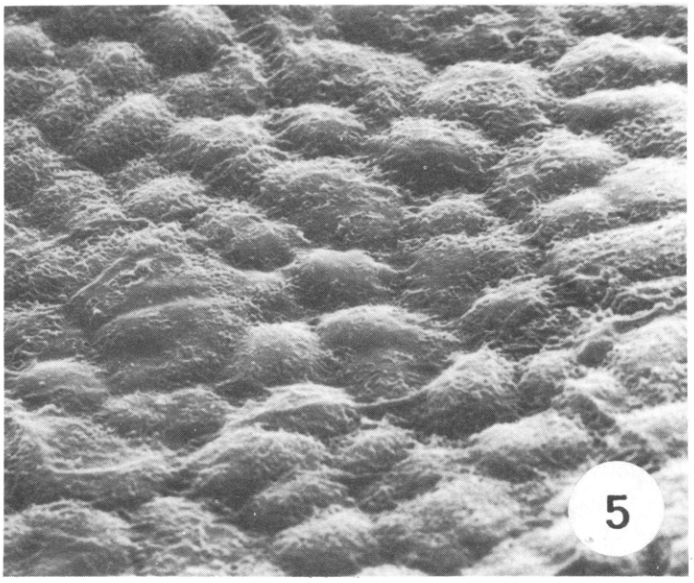


Fig. 5. Scanning electron micrograph of a cuticle from an apple fruit harvested in August. Note the development of the fine wax structure. Magnification x650.

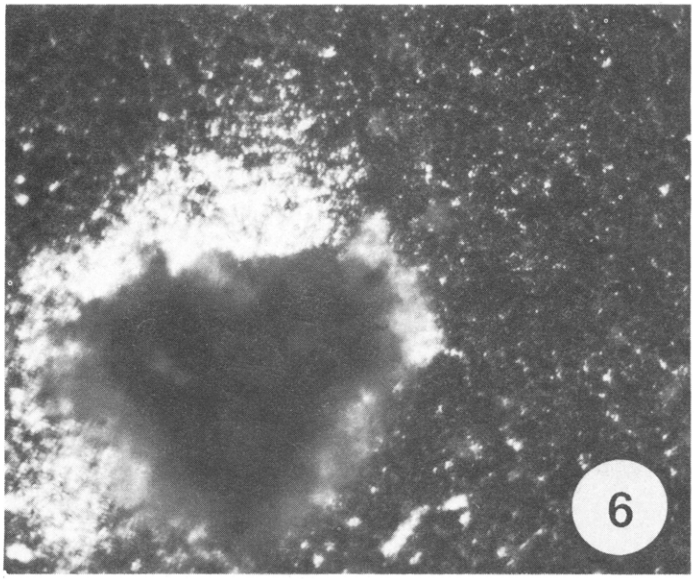


Fig. 6. Polarized light Micrograph of an agar mounted cuticle. Note the lenticel (dark structure in center of photo) with the Ca oxalate crystal beneath, and the small deposits occurring in other areas of the cuticle. Magnification x42.

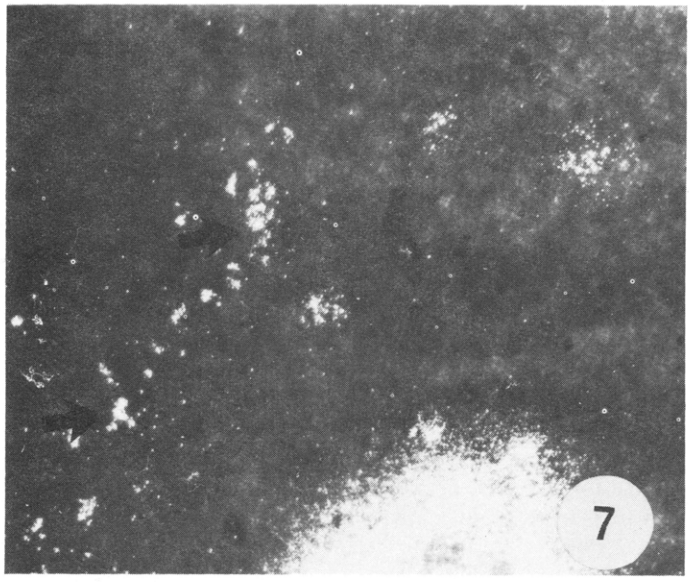


Fig. 7. Polarized light micrograph of Ca oxalate deposits in agar (cuticle has been removed). Bottom center: large crystal complex corresponding to a lenticel. Note the deposits of crystal (arrows) in other discrete areas corresponding to a russeted area of the apple cuticle. Magnification x33.

showed that there was no significant difference in permeability of cuticles from fruit harvested in August and September (Table 1). The permeability of cuticles isolated in July, August, and September and having exposed lenticels differed, but due to the high variability, the differences were not significant. Nevertheless, permeability tended to increase from August to September.

In the Ca localization studies, the wax seals effectively prevented leakage of Ca around the periphery of each cuticle. This assured that any Ca oxalate crystal formation observed on the agar was due solely to Ca penetration through the cuticle. The lenticel was found to be a major site of Ca penetration (Fig. 6).

Table 1. Permeability coefficients (k) of cuticles with and without lenticels exposed.

Harvest date	Avg no. of lenticels exposed/10 cm^2	Permeability coefficients (k) $\times 10^{-8}$ cm/sec	
		Lenticels exposed	No lenticels exposed
July	52	7.31 ± 1.41 NS[z]	not determined
August	40	3.02 ± 0.33 NS	1.04 ± 0.097 NS
September	35	14.13 ± 5.68 NS	1.47 ± 0.632 NS

[z]No significant difference at the 5% level. Differences between values of cuticles with and without lenticels exposed are significant at the 5% level.

A large variation was observed in the relative amounts of Ca penetration among different lenticels. Other surface irregularities, such as russetted areas and cracks, contributed to cuticular Ca penetration (Fig. 7).

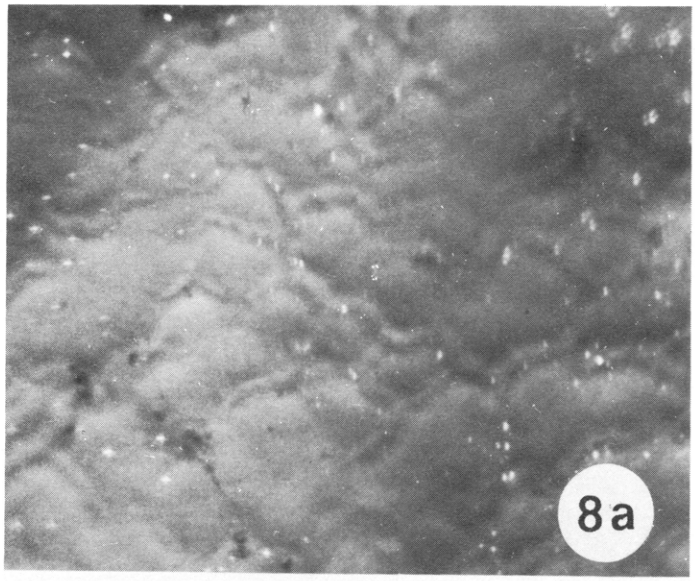
Small crystals of Ca oxalate were found to be associated with a reticulate pattern of indentations occurring in the agar (Fig. 8a). The pattern appeared to correspond with the reticulate pattern often observed in cuticles from fruit harvested in September (Fig. 8b).

Discussion

The pathway of Ca diffusion through the cuticle constitutes an important pathway in Ca penetration. To penetrate the cuticular structure, a Ca ion must traverse an outer waxy epicuticular layer, the underlying cutin matrix and the hydrophilic pectin and epidermal cell wall regions (28). It was observed in this study that, even though the total cuticular thickness increased significantly from August to September, there was no significant difference in the rate of Ca diffusion across crack-free cuticles isolated during these 2 months. A poor correlation between cuticle thickness and penetration has been reported by others as well (15, 23). It would therefore appear that a diffusion rate limiting fraction of the cuticle was formed before the fruit matured (7).

Various reports claim the epicuticular wax layer to be the major barrier to spray penetration. Schonherr and Bukovac (27) noted that mechanical disruption of the epicuticular wax layer resulted in increases in cuticular penetration. Reed and Tukey (23) reported that differences in the permeability of plant cuticles are more closely correlated with the interaction of cuticular thickness and percentage of wax content. If diffusion preferentially occurred in discrete areas of the apple cuticle as opposed to a uniform penetration, then diffusion must be facilitated by one or more of the following: a pore or similar structure; areas with a thinner wax layer; areas having a different chemical composition which would allow greater diffusion; areas of modified epicuticular wax structure; or, by a combination of any of these.

Schonherr and Bukovac (27) were able to show pathways of ion movement occurring along the anticlinal cell walls of *Allium* bulb scales. Although good contact between the cuticle and the agar was obtained in this study, no such pattern of Ca oxalate deposition was observed after an 18 hr incubation period. It can be calculated from the results of the diffusion study that in an 18 hr incubation period, an average of less than 5 parts per million of Ca diffused through a single cuticle. It is possible that pathways of diffusion similar to those described previously are involved in Ca transport through apple cuticles, but that the incubation period was not long enough to allow sufficient penetration for detection.



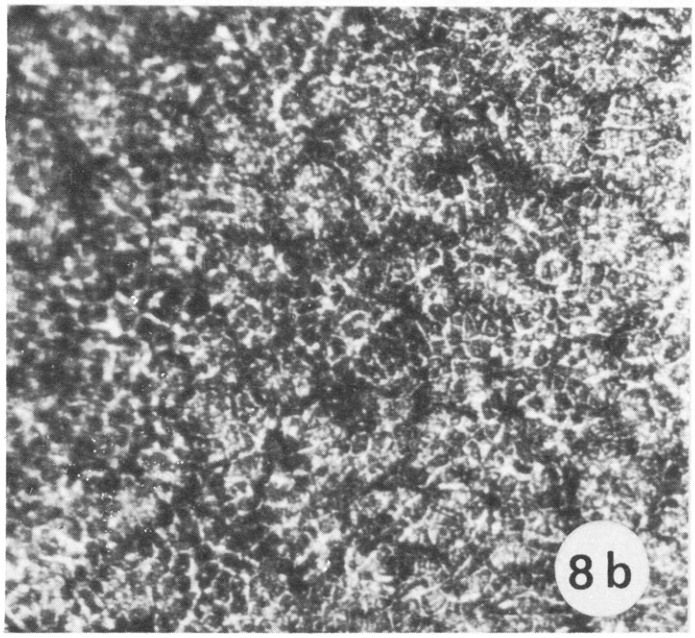


Fig. 8. **A**. Polarized light micrograph of Ca oxalate deposits in agar. Note that the crystal deposits occur in discrete areas corresponding to the reticulate pattern of indentations in the agar. Magnification x45. **B**. Light micrograph of a cuticle from an apple fruit harvested in September. Note that the reticulate pattern of cracking is similar to the pattern seen in the agar of the top photo. Magnification x45.

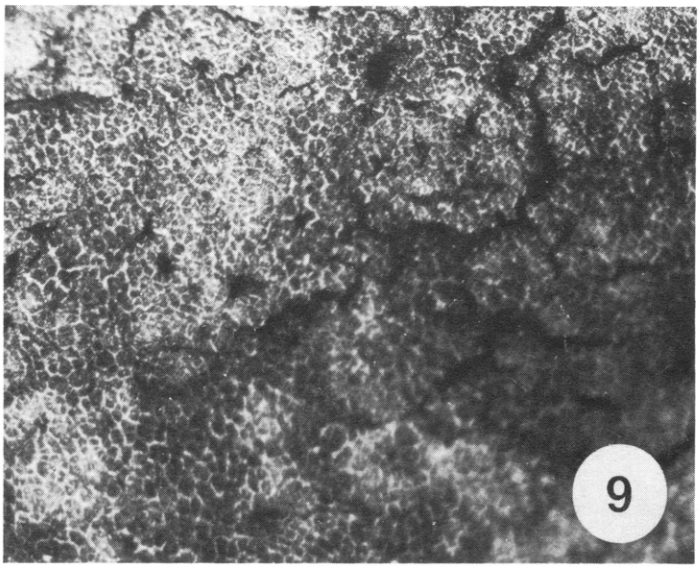


Fig. 9. Light micrograph of a cuticle isolated from fruit harvested in September. Note the formation of surface cracks. Magnification x29.

Cracks and other breaks in the surface of the cuticle may have an important effect in Ca penetration. Several researchers have reported cracks in the cuticle of apple fruit (3, 8, 14, 30). Tetley (30) suggested that cracks occur on the apple surface in weak areas such as stomata and hair bases. These points crack under pressure built up during fruit expansion. Tetley (30) further observed that surface cracks that penetrate the epidermis form a phellogen layer. Meyer's study (14) of Golden Delicious apple cuticles showed that crack width and number increase during fruit development.

Based on light micrographs alone, it was observed in this investigation that cuticles isolated from fruit harvested in September of 1982 had far more cracks than did fruit harvested in September of 1981. Growing conditions would seem to be an important factor in the development of cracks as suggested by Faust and Shear (3). In 1982, very few cracks were noted in August, but by September the majority of the cuticles exhibited cracking to some degree.

The localization study showed the lenticel to be the most common site of Ca penetration, even though some lenticels were observed to allow little or no cuticular penetration. The diffusion study using cuticles both with and without exposing the lenticels further demonstrated the marked effect lenticels may have on Ca penetration under saturated conditions where a Ca solution is in constant contact with the lenticel. Postharvest Ca treatments are successful in creating similar saturated conditions to one degree or another. Lee and Dewey (9) reported that dip solutions enter apple fruit primarily through the lenticel. The use of additives, such as food thickeners in dip solutions, has been found to be effective in increasing Ca levels further by maintaining a film of Ca solution in contact with the apple cuticle for extended periods of time (12). Although these methods of Ca treatment increase Ca uptake significantly in postharvest fruit, excessive fruit damage has discouraged their use on a large scale (5, 24). For this and other reasons, Ca sprays have gained wide acceptance.

Although the lenticel is reported to play a significant role in the uptake of Ca sprays (10), direct evidence is lacking. Field sprays of Ca result in a nonsaturated condition. The lenticels of a fruit have a relatively sparse distribution and therefore may come into direct contact with spray droplets less frequently than cracks in the cuticle surface. The role of lenticels in Ca spray absorption may be relatively less important than in absorption of postharvest dip solutions. Structural hindrance, as reported for stomatal structures (26), also may play a role in reducing spray penetration through the lenticel in unsaturated conditions.

The development of cracks and other surface irregularities during the latter part of the growing season may combine to play a significant role in Ca spray penetration into apple fruit. Late summer and early fall development of cracks may contribute to late season Ca sprays being more effective than early sprays (4, 5, 11, 25). The varying amounts of cracking observed from year to year in the cuticle of apple fruit may not only

account for the varied results of Ca sprays often observed between years, but also may implicate the relative importance of cracking as a pathway of Ca uptake.

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